REMARKS

Claims 15-16, and 19-20 are cancelled with this amendment. Claims 11, 17, and 18 have been amended. New claim 29 is added. Claims 11-14, 17-18 and 29 are now pending in this application. Support for the amendments is found in the existing claims and the specification as discussed below. Accordingly, the amendments do not constitute the addition of new matter. Applicant respectfully requests the entry of the amendments and reconsideration of the application in view of the amendments and the following remarks.

Double patenting

Claims 11, 12, and 19 are provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-8 of copending Application No. 10/581,568.

Applicant respectfully requests that the provisional double patenting rejection be held in abeyance until allowable subject matter is indicated.

Rejection under 35 U.S.C. § 103(a)

Claims 11-20 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Evans, et al. (US 2003/0236573 A1) and further in view of Gordinier, et al. (U.S. 5,599,558) and further in view of Ozek, et al. (Journal of Burn Care and Rehabilitation, pp. 65-69, January/February 2001).

Applicant and Applicant's representative thank Examiners Shah and Mondesi for the helpful interview on July 22nd which is summarized herein.

Claim amendments

All of the claims are now limited to a treatment by injection. Claims not directed to treatment of cancer by injection of a composition which includes platelet releasate have been cancelled. The present claims are directed to treatment of cancer. It is clear from the specification that the invention is applicable to a broad range of cancer types. See paragraphs 0003, 0010, 0102-0104, and 0125. Support for new claim 29 is found in paragraph 0103.

Unexpected results of claimed method

Applicant presents the attached Declaration by the inventor which shows the effectiveness of the claimed method in cancer treatment. The Declaration corresponds to the Power Point presented at the July 22nd interview.

The cancer cells chosen for the experiment were glioblastoma cells which is an aggressive brain cancer. Patients with this form of brain cancer have a median survival time of 3 months if no treatment is provided. As can be seen in Figure 1 of the Declaration, human glioblastoma cells have an undifferentiated, cancer-like morphology. After 10 days culture in the presence of 10% platelet releasate, the morphology has normalized (Figure 2). A possible astrocyte is show in Figure 3.

The platelet releasate-treated cancer cells appear to actually revert to a normal morphology as indicated by Figures 2 and 3. This result is even more unexpected in view of the art-accepted view of the role of growth factors in promoting cancer proliferation discussed below.

Treatment of cancer with PRP is counterintuitive

It would be counterintuitive for one of ordinary skill in the art to treat any cancer with platelet releasate because platelet releasate contains growth factors such as vascular endothelial growth factor (VEGF) (see present specification at paragraphs 0057 to 0096, particularly paragraph 0096). Such growth factors are known to promote angiogenesis needed for proliferation of cancer cells. In contrast to the present invention, the art teaches drugs which inhibit growth factors, such as VEGF, for cancer treatment. Anti-cancer drugs have been designed to inhibit growth factors such as VEGF.

Avastin® (Bevacizumab) is an antibody against VEGF which is approved for colon and non-small cell lung cancer (NSCLC) (see Annex 2). Tamoxifen is effective to treat estrogen-dependent breast cancer. It has been found that tamoxifen decreases extracellular levels of VEGF (see Annex 3). Both of these drugs treat cancer by lowering effective levels of VEGF. Accordingly, one of ordinary skill in the art in developing a treatment for cancer would more likely look for drugs to inhibit growth factors found in platelets such as VEGF. Use of a composition containing growth factors to treat cancer is counterintuitive.

The references as a whole do not suggest treatment of cancer with platelet releasate

The Office Action states that "Gordinier et al. teach treatment of chronic non-healing wounds i.e. cancer (as evidenced by Ozek et al that most chronically ulcerating wounds changes to cancer...)" (Office Action, page 6, first partial paragraph).

As discussed during the interview (summarized herein), Applicant disagrees that most chronically ulcerating wounds change to cancer and also that the disclosure of Gordinier, et al. would suggest treatment of cancer with platelet releasate to one of ordinary skill in the art.

As indicated by the attached Abstract by Copcu (Annex 1A), Marjolin's ulcer is a rare malignancy with a frequency of 0.77 to 2% for burn scars. Furthermore, the carcinoma takes a long time to develop. The attached references indicate 35 years (Annex 1A), and 43 years (Annex 1B). Most chronically ulcerating wounds do NOT change to cancer in contrast to the assertion in the Office Action and, if they do, this occurs decades after the wound has become chronic.

Accordingly, one of ordinary skill in the art reading Gordinier, et al. would not extrapolate from the commonly occurring types of chronic wounds such as venous ulcers, diabetic ulcers and pressure ulcers as taught by Gordinier in Table 1, subsections A, B, and C (col. 4, line 59 to col. 5, line 1) to a rare and slow-forming form of cancer more commonly associated with burn scars. Accordingly, one of ordinary skill in the art reading Gordinier's disclosure of treatment of chronic wounds would **not** understand use of platelet releasate to treat any cancer at all. Gordinier provides an extensive list of conditions that may be treated with platelet releasate (cols. 4-7). Notably, treatment of neither "cancer" nor "tumor" is mentioned.

In view of Applicant's amendments, arguments, Annex 1-3 and the attached Declaration from the inventor, reconsideration and withdrawal of the above ground of rejection is respectfully requested.

No Disclaimers or Disavowals

Although the present communication may include alterations to the application or claims, or characterizations of claim scope or referenced art, Applicant is not conceding in this application that previously pending claims are not patentable over the cited references. Rather,

any alterations or characterizations are being made to facilitate expeditious prosecution of this application. Applicant reserves the right to pursue at a later date any previously pending or other broader or narrower claims that capture any subject matter supported by the present disclosure, including subject matter found to be specifically disclaimed herein or by any prior prosecution. Accordingly, reviewers of this or any parent, child or related prosecution history shall not reasonably infer that Applicant has made any disclaimers or disavowals of any subject matter supported by the present application.

CONCLUSION

In view of Applicants' amendments to the claims and the foregoing Remarks, it is respectfully submitted that the present application is in condition for allowance. Should the Examiner have any remaining concerns which might prevent the prompt allowance of the application, the Examiner is respectfully invited to contact the undersigned at the telephone number appearing below.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: Aug. 31, 2009

By: 

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ANNEX 1A

Plast Reconstr Surg. 2009 Jul;124(1):156e-64e. Wolters Kluwer | Lippincott Williams & Wilkins Links

I. Marjolin's ulcer: a preventable complication of burns?

Copcu E.

Plastic, Reconstructive, and Aesthetic Surgery Department, Medical Faculty, Adnan Menderes University, Aydin, Turkey. ecopcu@adu.edu.tr

LEARNING OBJECTIVES: After studying this article, the participant should be able to: 1. Discuss the clinical features of Marjolin's ulcer. 2. Identify the risk factors for the development of Marjolin's ulcer. 3. Develop a surgical management plan for the treatment of Marjolin's ulcer. SUMMARY: Marjolin's ulcer is a rare and aggressive cutaneous malignancy that arises on previously traumatized and chronically inflamed skin, especially after burns. This clinical condition was first described by Marjolin in 1828. The term "Marjolin's ulcer" has been generally accepted to refer to a long-term malignant complication of the scars resulting from burns. However, vaccination, snake bites, osteomyelitis, pilonidal abscesses, pressure sores, and venous stasis may also induce this tumor. Clinically, reports suggest that atrophic and unstable scars tend to develop into cancer. Various etiological factors have been implicated in the condition, including toxins released from damaged tissues, immunologic factors, cocarcinogens, and miscellaneous factors such as irritation, poor lymphatic regeneration, antibodies, mutations, and local toxins. The incidence of burn scars undergoing malignant transformation has been reported to be 0.77 to 2 percent. All parts of the body can be affected, but the extremities and the scalp are most frequently affected. There are two variants: acute and chronic. In the former, the carcinoma occurs within 1 year of the injury. The chronic form is more frequent and malignancy tends to develop slowly, with an average time to malignant transformation of 35 years. Although many different cell types can be seen in these lesions, the major histological type is squamous cell carcinoma. Marjolin's ulcers are generally considered as very aggressive tumors with a higher rate of regional metastases; radical excision is the treatment of choice, but there is no consensus on lymph node dissection. Marjolin's ulcer can be insidious and often leads to a poor prognosis, and deaths from Marjolin's ulcer are not uncommon. Meticulous wound care is a crucial step in prevention of these lesions.

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ANNEX 1B

Rev Chir Orthop Reparatrice Appar Mot. 2007 Feb;93(1):63-71. Full text on EM|consulte Free access in english Links

I. [Marjolin's ulcer in chronic osteomyelitis: seven cases and a review of the literature]

[Article in French]

Bauer T, **David T**, **Rimareix F**, **Lortat-Jacob A**.

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PURPOSE OF THE STUDY: Malignant degeneration of chronic wound inflammation is a rare complication which almost always develops late. Unstable wounds and scar tissue related to chronic osteitis can degenerate after a long period of chronic inflammation. We report seven cases. CASE REPORTS: Seven patients presented squamous-cell carcinoma of the skin which had developed on wounds related to deep bone infections. Three patients had chronic bone infections subsequent to posttraumatic osteitis, two after hematogenous osteomyelitis, one after osteitis which developed on a zone of radiation-induced necrosis, and one after a deep burn was complicated by osteitis. The skin lesions developed over a period of 43 years on average before the diagnosis of malignant degeneration was established. Most of the lesions presented as budding malodorous ulcers. The pathological diagnosis was spinocellular squamous-cell carcinoma in five cases and verrucous squamous-cell carcinoma in two. Conservative treatment with wide resection and flap cover was attempted in all seven patients. RESULTS: Treatment failed in four patients and three required amputation. One patient died two years after amputation with local recurrence and metastatic dissemination to the brain. DISCUSSION: The diagnosis of malignant degeneration requires pathological proof. Biopsy material should be obtained whenever there is a modification leading to the development of a fistula or the formation of a scar tissue over a focus of chronic osteitis. Prevention requires adapted treatment of chronic bone infections, avoiding directed wound healing which can lead to fragile unstable scar tissue subject to degeneration.

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Annex 2

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A Randomized Trial of Bevacizumab, an Anti–Vascular Endothelial Growth Factor Antibody, for Metastatic Renal Cancer

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ABSTRACT

BACKGROUND

Mutations in the tumor-suppressor gene *VHL* cause oversecretion of vascular endothelial growth factor by clear-cell renal carcinomas. We conducted a clinical trial to evaluate bevacizumab, a neutralizing antibody against vascular endothelial growth factor, in patients with metastatic renal-cell carcinoma.

METHODS

A randomized, double-blind, phase 2 trial was conducted comparing placebo with bevacizumab at doses of 3 and 10 mg per kilogram of body weight, given every two weeks; the time to progression of disease and the response rate were primary end points. Crossover from placebo to antibody treatment was allowed, and survival was a secondary end point.

RESULTS

Minimal toxic effects were seen, with hypertension and asymptomatic proteinuria predominating. The trial was stopped after the interim analysis met the criteria for early stopping. With 116 patients randomly assigned to treatment groups (40 to placebo, 37 to low-dose antibody, and 39 to high-dose antibody), there was a significant prolongation of the time to progression of disease in the high-dose–antibody group as compared with the placebo group (hazard ratio, 2.55; $P<0.001$). There was a small difference, of borderline significance, between the time to progression of disease in the low-dose–antibody group and that in the placebo group (hazard ratio, 1.26; $P=0.053$). The probability of being progression-free for patients given high-dose antibody, low-dose–antibody, and placebo was 64 percent, 39 percent, and 20 percent, respectively, at four months and 30 percent, 14 percent, and 5 percent at eight months. At the last analysis, there were no significant differences in overall survival between groups ($P>0.20$ for all comparisons).

CONCLUSIONS

Bevacizumab can significantly prolong the time to progression of disease in patients with metastatic renal-cell cancer.

From the Surgery Branch (J.C.Y., L.H., R.M.S., P.H., D.J.S., S.L.T., S.A.R.), the Biostatistics and Data Management Section (S.M.S.), and the Cancer Therapy Evaluation Program (H.X.C.), National Cancer Institute, Bethesda, Md. Address reprint requests to Dr. Yang at Rm. 2B-37, Bldg. 10, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892, or at james_yang@nih.gov.

N Engl J Med 2003;349:427-34.

STUDIES OF THE HEREDITARY FORM OF clear-cell renal carcinoma, which occurs in the von Hippel–Lindau syndrome, led to the identification of the von Hippel–Lindau tumor suppressor gene (*VHL*). The gene is mutated both in hereditary renal-cell carcinoma (where one mutation is a germ-line mutation) and in most cases of sporadic clear-cell renal carcinoma (where both alleles have acquired mutations or deletions).[1,2] One consequence of these mutations is the overproduction of vascular endothelial growth factor through a mechanism involving hypoxia-inducible factor α.[3-7] In addition, both *VHL*-deficient mice and vascular endothelial growth factor–knockout mice die in utero from defective vasculogenesis.[8,9] Thus, by its regulation of vascular endothelial growth factor, the von Hippel–Lindau protein is tightly linked to angiogenesis. Vascular endothelial growth factor stimulates the growth of endothelial cells and appears to be a central factor in angiogenesis, particularly during embryogenesis, ovulation, wound healing, and tumor growth.[10]

Studies of human tumor xenografts in immunodeficient mice showed that neutralization of vascular endothelial growth factor inhibited the growth of a variety of model tumors.[11,12] Presta and colleagues "humanized" the murine antibody used in these studies, A.4.6.1, by placing its complementarity-determining (antigen-binding) regions into a human IgG1 constant-region framework and modifying further amino acid residues to optimize antigen binding.[13] In the resulting product, bevacizumab (or rhMAb-VEGF), 7 percent of the amino acids are from the murine antibody. In phase 1 testing, bevacizumab had a low toxicity profile in most patients, had a terminal elimination half-life of approximately 21 days, and did not induce antibodies to bevacizumab.[14] The severe toxic effects that occurred in the phase 1 trial were infrequent intratumoral bleeding (including fatal hemoptysis), pulmonary emboli, and peripheral venous thrombosis. We conducted a randomized, placebo-controlled phase 2 trial of bevacizumab in patients with advanced renal-cell carcinoma.

METHODS

PATIENTS

Patients with histologically confirmed renal cancer of the clear-cell type, measurable metastatic disease, and documented progression of disease were eligible for this study. Other requirements included an Eastern Cooperative Oncology Group (ECOG) performance status of 2 or lower and previous therapy with interleukin-2 (or contraindications to standard interleukin-2 therapy). The exclusion criteria were a history of central nervous system involvement, any other therapy or major surgery within the previous four weeks, a history of intratumoral bleeding, a serum creatinine level of more than 2 mg per deciliter (177 μmol per liter), a serum bilirubin level of more than 2 mg per deciliter (34 μmol per liter), and ischemic vascular disease.

All patients gave written informed consent. This protocol was approved by the institutional review board of the National Cancer Institute (NCI). The study was sponsored by the Cancer Therapy Evaluation program of the NCI, and bevacizumab was supplied by Genentech under a cooperative research and development agreement with the NCI. Trial design, data accrual (with the exception of assays for vascular endothelial growth factor and bevacizumab performed by Genentech on coded patient specimens), data analysis, and manuscript preparation were performed entirely by the authors.

The patients were evaluated by physical examination, magnetic resonance imaging of the brain, and complete computed tomographic scanning no more than one month before randomization, five weeks after the beginning of therapy, and then every two months for the first year of therapy and every three months for the second year of therapy.

A complete response was defined as the absence of all evidence of disease for at least a month. A partial response was defined as a decrease of at least 50 percent in the sum of the products of the maximal perpendicular diameters of measured lesions, lasting for a minimum of one month, with no progression of any lesion or appearance of new lesions. Minor and mixed responses were not included as responses.

Annual interim evaluations were performed by an independent data safety and monitoring board, and the method of O'Brien and Fleming was used to determine the threshold for statistical significance at each interim evaluation that would constitute grounds to recommend termination of the trial.[15] For the first year of the trial, this threshold was a P value of 0.0006 or less; for the second year, it was a P value of 0.015 or less; and for the third year, it was a P value of 0.047 or less. The estimated and actual accrual rates were similar enough that these proposed intervals did not require revision.

RANDOMIZATION AND TREATMENT

In this phase 2 study, the patients were stratified according to whether or not they had received interleukin-2 therapy and were then randomly assigned to receive either a vehicle-only placebo, 3 mg of bevacizumab per kilogram of body weight, or 10 mg of bevacizumab per kilogram. During all treatment and evaluations, neither the patients nor any participating health care personnel were aware of the treatment assignment. Based on pharmacokinetic modeling, treatment with bevacizumab began with one loading dose, in which 150 percent of the assigned dose was administered by intravenous infusion over a 2-hour period, and then, beginning one week later, the standard assigned dose was administered (by progressively shorter infusions that reached a minimum of 30 minutes) every two weeks. Plasma levels of vascular endothelial growth factor and serum levels of bevacizumab were measured. The plasma vascular endothelial growth factor assay used the 3.5.E.8 murine antibody for both capture and detection. This assay detects both free and bevacizumab-bound vascular endothelial growth factor equally, with a lower limit of detection of 40 pg per milliliter.

EVALUATION

For the purposes of end-point evaluation, the criteria for declaring tumor progression were the unequivocal appearance of new lesions; an increase of more than 25 percent in the product of the maximal perpendicular diameters of any measured lesion, as compared with base-line evaluation (or the smallest size subsequent to base line); or a tumor-related deterioration in ECOG performance status to 3 or more. For a declaration of progressive disease to be made, the lesions had to attain a minimal diameter of 1.5 cm (to ensure accurate measurement).

The indications for removing patients from the study and unblinding their treatment assignments were as follows. To permit adequate time for the initial assessment of the therapy while protecting patients with rapid disease progression who were assigned to placebo, the evaluation conducted five weeks after enrollment differed from subsequent evaluations. At five weeks, patients with increases of more than 2 cm in any lesion, a clinically significant deterioration in performance status, or new, severe symptoms (e.g., bone pain or nerve compression) were removed from the study. At all other evaluations, the indication for removal from the study was progressive disease. These different indications for removal from the study did not affect the end-point analyses, which were always based on tumor progression, as defined above.

STATISTICAL ANALYSIS

Using NCI Surgery Branch historical data from patients with no response to interleukin-2 therapy, we used the following criteria to estimate the sample size necessary to detect a doubling of the time to progression in patients receiving either dose of bevacizumab as compared with those receiving placebo: a 24-month accrual period, a 12-month evaluation period after the completion of accrual, a power of 80 percent, and an overall alpha of 0.05 to detect a doubling of the hazard ratio for each of the two primary comparisons (high-dose antibody vs. placebo and low-dose antibody vs. placebo). The calculation indicated that 40 patients per group would be required (50 were permitted, to allow for some patients who could not be evaluated).

The primary evaluation was based on the time from enrollment to disease progression; a secondary analysis examined the time to disease progres-

Table 1. Characteristics of Patients before Treatment.*

Characteristic	High-Dose Bevacizumab (N=39)	Low-Dose Bevacizumab (N=37)	Placebo (N=40)
Median age (yr)	53	54	53
Male sex (%)	74	84	68
ECOG performance status (no.)†			
0	30	30	31
1 or 2	9	7	9
Prior interleukin-2 therapy (no.)	37	34	37
Prior chemotherapy (no.)	10	7	8
Prior radiation therapy (no.)	8	6	12
Prior nephrectomy (no.)	35	33	38
Anemia (no.)	14	15	16
Hypercalcemia (no.)	12	18	14
Interval from diagnosis to randomization (no.)			
<1 yr	14	13	12
1–2 yr	8	6	9
>2 yr	17	18	19
Liver involvement (no.)	10	10	10
Bone involvement (no.)	2	3	6

* P>0.05 for all comparisons.
† ECOG denotes Eastern Cooperative Oncology Group. Higher performance-status numbers indicate greater impairment.

sion from the five-week assessment, in order to determine whether the effect of treatment was delayed and to ensure that small variations in the interval from the pretreatment evaluation to the time of randomization did not affect the uniform determination of the time to progression. Each P value was adjusted for the performance of two primary comparisons on the basis of treatment groups.

The time to progression and the overall response rate were the primary end points, and the analyses were performed on an intention-to-treat basis. Survival was declared a secondary end point, because patients whose disease progressed while they were receiving placebo were offered crossover either to 3 mg of bevacizumab per kilogram alone or to a combination of 3 mg of bevacizumab per kilogram and thalidomide. The time to progression of disease and survival were assessed with use of Kaplan–Meier curves and tested for significance by the log-rank test. Hazard ratios were determined with the Cox proportional-hazards model. All P values are two-tailed.

Table 2. Toxic Effects of Treatment.*

Effect	High-Dose Bevacizumab (N=39)	Low-Dose Bevacizumab (N=37)	Placebo (N=40)
		number	
Epistaxis	8†	5	1
Hypertension	14† (8†)	1	2
Fever without infection	4	1	0
Malaise	13	6	6
Hematuria	5†	1	0
Hyponatremia	3	4†	0
Proteinuria (≥1+ or ≥150 mg/24 hr)	25† (3)	15 (2)	15
Elevated alanine aminotransferase	4	2	0
Chest pain	2 (2)	0	0

* The table lists all toxic effects of any grade that occurred in at least 10 percent of patients receiving either dose of antibody and that were more frequent than in patients receiving placebo. The number of patients with grade 3 toxic effects is shown in parentheses (there were no grade 4 or 5 events; every bevacizumab-associated grade 3 toxic effect occurring in more than one patient is shown). Grade 3 hypertension was defined as hypertension not completely controlled by one standard medication. Grade 3 proteinuria was defined as urinary excretion of more than 3.5 g of protein per 24 hours. Other toxic effects were graded according to the National Cancer Institute Common Toxicity Criteria (version 2.0).

† Unadjusted P≤0.05 for the comparison with placebo (by chi-square test, or by Fisher's exact test if the expected frequency was less than 5).

RESULTS

Between October 1998 and September 2001, 116 patients were enrolled, of whom 108 had progressive disease during the course of the study. The median follow-up time from study entry was 27 months. Forty patients were randomly assigned to placebo, 37 to low-dose bevacizumab, and 39 to high-dose bevacizumab. All planned doses of the study drug were given unless grade 3 toxic effects occurred, in which case doses were withheld as specified by the study protocol. Only one patient (who was assigned to low-dose bevacizumab) was lost to follow-up after therapy. The three groups had similar demographic and clinical characteristics and laboratory results (Table 1). All patients received at least one dose of the assigned drug, and 114 of the 116 patients underwent at least one planned follow-up evaluation (evidence concerning disease progression was available for the remaining 2 patients).

There were no life-threatening toxic effects (grade 4, major organ) or deaths possibly related to bevacizumab (Table 2). Hypertension and asymptomatic proteinuria were associated with bevacizumab therapy (Table 2). Of 13 patients with grade 2 or 3 hypertension, 7 (54 percent) had grade 2 or 3 proteinuria; of 63 patients with grade 0 or 1 hypertension, 10 (16 percent) had grade 2 or 3 proteinuria ($P=0.007$ by Fisher's exact test). None of these patients, or any other patient, had renal insufficiency. Hypertension and proteinuria uniformly decreased after the cessation of therapy, but death from renal cancer, the slow rate of correction of hypertension and proteinuria, and the commencement of other therapies prevented the documentation of complete resolution of these toxic effects in all but one patient.

There were no episodes of grade 4 hypertension during randomized therapy, but in one patient who was initially assigned to placebo, hypertension with coma developed after the patient crossed over to low-dose bevacizumab plus thalidomide. These complications resolved completely after therapy was stopped. Typically, hypertension during the study was treated by the patients' private physicians with standard regimens for essential hypertension. Among all bevacizumab-treated patients who required therapy for newly diagnosed hypertension (for whom the dates of onset could be most accurately determined), the median interval from the first dose of bevacizumab to the onset of hypertension was 131 days (range, 7 to 316). Grade 1 or 2 hemoptysis developed in four patients (one receiving

high-dose bevacizumab, one receiving low-dose bevacizumab, and two receiving placebo), and one patient receiving placebo had a pulmonary embolus.

At the second interim evaluation (which analyzed the data on 110 patients), the NCI data safety and monitoring board recommended closure of accrual on the basis of the difference between the placebo and high-dose bevacizumab groups in the time to progression of disease. According to intention-to-treat analysis, progression-free survival in the group receiving 10 mg of bevacizumab per kilogram (with a median time to progression of 4.8 months) was significantly longer than that in the placebo group (with a median time to progression of 2.5 months, P<0.001 by the log-rank test) (Fig. 1A). The difference between the time to progression of disease in the group receiving 3 mg of bevacizumab per kilogram (median time, 3.0 months) and that in the placebo group was of borderline significance (P=0.041 by the log-rank test) (Fig. 1B).

The planned analysis of progression from the five-week assessment yielded the same results. The percentages of patients assigned to high-dose bevacizumab, low-dose bevacizumab, and placebo who had no tumor progression were 64 percent, 39 percent, and 20 percent, respectively, four months after randomization and 30 percent, 14 percent, and 5 percent eight months after randomization. A Cox proportional-hazards model yielded hazard ratios for the time to progression of disease of 2.55 among patients given high-dose bevacizumab (P<0.001) and 1.26 among those given low-dose bevacizumab (P=0.053), as compared with those given placebo.

Only four patients had objective responses (all of which were partial responses), and all of these had received high-dose bevacizumab; thus, the response rate for high-dose bevacizumab was 10 percent (95 percent confidence interval, 2.9 to 24.2 percent). One patient had a partial response for the maximal treatment period of two years. This patient then stopped therapy, had a relapse six months later, and is currently having a second partial response after retreatment under a compassionate exemption (Fig. 2). Another patient treated for two years had a sustained minor response, had a relapse after stopping therapy, and had another minor response after being retreated.

Measurements of plasma vascular endothelial growth factor were available for 113 patients. Of these, 76 had a base-line level below the lower limit of detection (40 pg per milliliter). There were no significant associations between a detectable pretreatment level of vascular endothelial growth factor and the clinical response or the time to progression in either bevacizumab group (all P values were greater than 0.20). However, the limited sensitivity of the assay does not permit the definitive conclusion that there is no correlation between the base-line plasma level of vascular endothelial growth factor and the clinical response or the time to progression. After antibody therapy was started, the plasma levels of vascular endothelial growth factor rose steadily (the assay measures both free and antibody-bound vascular endothelial growth factor). After 5 weeks

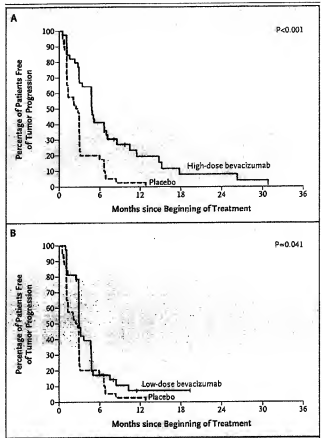

Figure 1. Kaplan–Meier Analysis of Survival Free of Tumor Progression for Patients Receiving High-Dose Bevacizumab (Panel A) or Low-Dose Bevacizumab (Panel B), as Compared with Placebo.
The high dose of bevacizumab was 10 mg per kilogram of body weight. The low dose of bevacizumab was 3 mg per kilogram. Doses were given every two weeks. P values were calculated by the log-rank test.

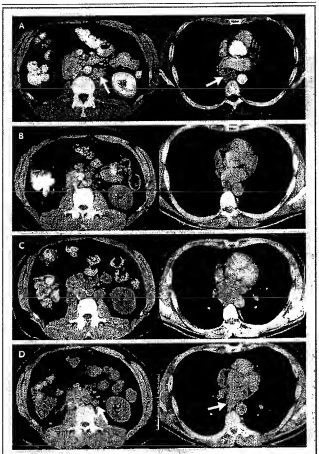

Figure 2. Serial Radiographs of a Patient Treated with High-Dose Bevacizumab.
Panel A shows the pretreatment assessment (arrows indicate lymph-node metastases). Panel B shows a radiograph obtained two years later, when treatment was stopped during a partial response. Panel C shows relapse of tumor six months thereafter. Panel D shows a second partial response 3 months after therapy was restarted, which is ongoing at more than 18 months as of this writing.

and 13 weeks of therapy, all bevacizumab-treated patients had detectable plasma levels of vascular endothelial growth factor. The median levels were 196 and 246 pg per milliliter, respectively, for patients receiving high-dose bevacizumab and 155 and 170 pg per milliliter for patients receiving low-dose bevacizumab. The percentages of patients assigned to placebo who had undetectable plasma levels of vascular endothelial growth factor at base line, 5 weeks, and 13 weeks were 66 percent, 67 percent, and 75 percent, respectively. Patients receiving low-dose bevacizumab had mean (±SE) peak and trough serum levels of bevacizumab of 101±9 and 39±3 μg per milliliter, respectively; patients receiving high-dose bevacizumab had mean peak and trough levels of 392±24 and 157±13 μg per milliliter, respectively. In both groups, the trough levels were above that needed to abolish detectable free vascular endothelial growth factor in the plasma of patients in previous phase 1 studies.[14]

At the most recent analysis, in February 2003, 19 of 116 patients (16 percent) were alive, and there were no significant differences in survival between the treatment groups (all P values were greater than 0.20) (Fig. 3). The complete radiographic records of 113 patients (3 were no longer complete at the time of audit) were blindly audited by a team of extramural radiologists under the supervision of the Cancer Therapy and Evaluation Program of the NCI. The prolongation of time to progression of disease was confirmed radiologically.

DISCUSSION

We selected vascular endothelial growth factor as a target for treatment of clear-cell kidney cancer because mutations in the von Hippel–Lindau tumor-suppressor gene, which probably cause most sporadic clear-cell kidney cancers, result in overproduction of this growth factor by the tumors. In our study, the aim was to neutralize vascular endothelial growth factor with a humanized monoclonal antibody (bevacizumab) in patients with metastatic clear-cell renal cancer. Using a randomized, double-blind, placebo-controlled design, we found that the time to tumor progression was prolonged by a factor of 2.55 in patients given 10 mg of bevacizumab per kilogram every two weeks, as compared with patients in the placebo group. Survival was not a primary end point in this trial, which allowed patients to cross over from placebo to bevacizumab therapy at the time of disease progression. Indeed, the survival of bevacizumab-treated patients was not significantly different from that of the patients receiving placebo.

During bevacizumab therapy, the plasma level of vascular endothelial growth factor rose. It is important to note that the assay we used measured both free and antibody-bound vascular endothelial growth factor. The explanation for this increase and its clinical significance are unknown, but it might have been due to diminished clearance of bevaci-

zumab-bound, inactive vascular endothelial growth factor or to an antibody-mediated blockade of the binding of vascular endothelial growth factor to its receptors.

Hypotheses about the mechanism responsible for the delay we observed in tumor progression are based on in vitro data, the results of treatment of human tumor xenografts in immunodeficient mice, and studies of human renal cancer. These data suggest that the antitumor effects of the antibody against vascular endothelial growth factor are due to inhibition of angiogenesis. Both in vitro and in tumor xenografts, vascular endothelial growth factor has potent angiogenic activity, which is inhibited by neutralizing antibodies to vascular endothelial growth factor; the result is a decrease in tumor blood flow and microvessel densities.[11] Human clear-cell renal cancers have significantly higher microvessel counts than non–clear-cell renal cancers, and these counts are correlated with the expression of vascular endothelial growth factor.[16] Endothelial cells and hematopoietic cells (but not renal cancer cells) are the predominant cells that express receptors for vascular endothelial growth factor, but the inhibition of the growth of human tumor xenografts in immunodeficient mice argues against contributions from an immunologic mechanism. For all these reasons, the inhibitory effect of bevacizumab on the growth of clear-cell renal cancer is likely to be due to its antiangiogenic action.

Antiangiogenic strategies for the treatment of cancer have generated widespread enthusiasm based on promising in vitro and preclinical studies. The concepts that growing tumors require the manufacture of new blood vessels and that very little of the rest of the normal adult body has such a requirement have led to the belief that there is valuable therapeutic potential in this area. Early clinical studies of antiangiogenic compounds such as endostatin, TNP-470, and thalidomide were not designed to assess their clinical efficacy.[17,18] In retrospect, only a randomized assessment of a time-to-progression end point could have demonstrated the activity of bevacizumab in renal cancer. Reliance on major response rates would have resulted in the conclusion that this drug was ineffective. Nevertheless, without a demonstration of improved overall survival, this single-agent trial serves primarily as a proof of principle and the basis for further investigation.

The magnitude of the clinical benefit of bevacizumab in this trial was small. The differences in the time to the progression of disease between the high-dose bevacizumab group and the placebo group was only a few months. Nevertheless, the likelihood is high that this difference was due to true biologic activity. The lack of an overall survival benefit in this trial and the small size of the increase in the time to progression may reflect the crossover design and the rigorous indications for declaring progression and removing a patient from the study (an increase in diameter of any single lesion by as little as 12 percent could constitute tumor progression). Some patients left the study with only small new lesions or mixed responses, but often with minimal or no increase in the size of preexisting tumors. In fact, 23 patients treated with high-dose bevacizumab showed no net increase in the size of index lesions from base line to the time of tumor progression. Tumor progression in these patients was typically based on the appearance of small new lesions or an increase in the size of some lesions that was offset by regression in other lesions. It would be worthwhile to determine survival in patients allowed to continue to receive bevacizumab despite tumor progression.

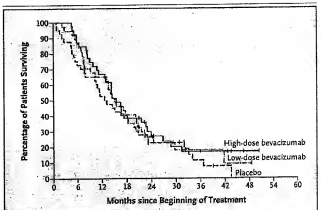

Figure 3. Overall Survival of Patients Receiving Placebo, Low-Dose Bevacizumab, or High-Dose Bevacizumab.
There were no significant differences among the treatment groups.

Future treatments for renal cancer that target angiogenic mechanisms should consider pathways other than that mediated by vascular endothelial growth factor. There are other proteins in the local microenvironment of some tumors that can promote angiogenesis. For example, fibroblast growth factor 5, which has angiogenic activity, is secreted

by most renal cancers,[19] suggesting that combinations of bevacizumab and inhibitors of members of the fibroblast growth factor family may have promise for treatment of this disease. It is likely that the future of antiangiogenic therapy will require a rational combination of inhibitors, directed by a better understanding of the biology of each individual type of cancer.

We are indebted to the Surgery Branch research nurses and immunotherapy fellows, the day hospital nursing staff, Don White, Maria Merino, W. Marston Linehan, Richard Klausner, Gwen Fyfe, and William Novotny for their invaluable assistance in the conduct of this study.

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Annex 3

[CANCER RESEARCH 63, 8742–8748, December 15, 2003]

Tamoxifen Inhibits Secretion of Vascular Endothelial Growth Factor in Breast Cancer *in Vivo*

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ABSTRACT

Vascular endothelial growth factor (VEGF) is considered a key mediator of tumor angiogenesis, including neovascularization in human breast cancer. High tissue VEGF levels appear to correlate with poor prognosis and decreased overall survival in node-positive and node-negative breast cancer patients. Hormonal regulation of VEGF expression has been demonstrated, and some reports indicate that tamoxifen, a partial estrogen receptor agonist, increases VEGF mRNA in breast cancer cells. These results appear to contradict the efficacy of tamoxifen as an adjuvant for estrogen-dependent breast cancer, yet clinical data show that tamoxifen prevents metastasis and increases overall survival. In this study, we confirmed previous studies showing that intracellular levels of VEGF *in vitro* increased in response to tamoxifen to levels similar to those observed after estrogen treatment. To further study hormonal effects on the release of VEGF, we used microdialysis to sample the extracellular space, where VEGF is biologically active, in solid tumors *in situ*. We show for the first time that tamoxifen decreased extracellular VEGF *in vivo* in solid MCF-7 tumors in nude mice. These *in vivo* findings were confirmed *in vitro* where extracellular VEGF in the cell culture medium was decreased significantly by tamoxifen treatment. Furthermore, we illustrate that microdialysis is a viable method that may be applied in human breast tissue to detect soluble VEGF *in situ* released by the tumor.

INTRODUCTION

Angiogenesis has been shown to be an essential factor for tumor growth and development of metastasis (1). Vascular endothelial growth factor (VEGF) is a key factor in promotion of tumor angiogenesis (1, 2). In breast cancer tissue, VEGF mRNA expression is increased compared with adjacent normal breast tissue (3). Moreover, high tissue VEGF levels appear to correlate with poor prognosis, and decreased overall survival for node-positive and node-negative breast cancer patients (4, 5). Several VEGF isoforms are produced from a single gene as a result of alternative splicing (6). The isoforms differ in their biological properties and in their abilities to bind heparan sulfate proteoglycans (2). VEGFs are bioactive as freely diffusible proteins in the extracellular space where they become available to endothelial cells, and in one report it has been suggested that the soluble isoforms have greater angiogenic and tumorigenic properties than the heparin-bound isoforms (7).

Estrogen has been shown to modulate angiogenesis in the female reproductive tract under physiologic and pathologic conditions, mainly via effects on endothelial cells (8, 9). Moreover, an estrogen-responsive element in the promoter region of the gene for VEGF has been identified (10). Estrogen exposure is considered a major risk factor for development of breast cancer, and the majority of breast cancers maintain their hormonal dependency (11–13). Therefore, strategies aiming at reducing the influence of estrogen on breast cancer cells have been developed. The most frequently used endocrine therapy for all of the stages of breast cancer is tamoxifen. Tamoxifen is a partial agonist of the estrogen receptor, and it has been shown that breast cancer cells exposed to tamoxifen increase VEGF mRNA expression (14, 15). This result would seem to contradict the efficacy of tamoxifen as an adjuvant for estrogen-dependent breast cancer. However, little is known about the hormonal regulation of VEGF, including the effects of tamoxifen on the levels of VEGF in the extracellular space.

Therefore, we have investigated effects of tamoxifen on VEGF secretion in human estrogen receptor-positive breast cancer cells *in vitro* and in solid tumors *in vivo*. In the present study, we show that the *in vitro* secretion of VEGF was inhibited by tamoxifen, whereas the intracellular levels of VEGF were increased by tamoxifen in the same manner as with estradiol. Moreover, we could verify, using microdialysis, that tamoxifen treatment decreased secretion of VEGF *in vivo* in solid breast cancer tumors in nude mice.

MATERIALS AND METHODS

Cells and Culture Conditions. MCF-7 cells (estrogen receptor-positive and progesterone receptor-positive; American Type Culture Collection, Manassas, VA) were used in all of the experiments. Cells were cultured in DMEM without phenol red supplemented with 2 mM glutamine, 50 IU/ml penicillin-G, 50 μg/ml streptomycin, and 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2. Cell culture medium and additives were obtained from Life Technologies, Inc. (Paisley, United Kingdom) if not otherwise stated. Before the experiments, cells were trypsinized (0.05% trypsin and 0.02% EDTA) and seeded into Petri dishes (Cosar, Cambridge, MA), 10,000 cells/cm^2. Cells were incubated for 1 day and then treated with or without 10^{-8} M estrogen (17β-estradiol; Apoteket, Umeå, Sweden), 10^{-6} M tamoxifen (Sigma, St. Louis, MO), or a combination of estradiol and tamoxifen. Hormones were added to the MCF-7 cultures in serum-free medium consisting of a 1:1 mixture of nutrient mixture F-12 (HAM) and DMEM without phenol red supplemented with 10 μg/ml transferrin (Sigma), 1 μg/ml insulin (Sigma), and 0.2 mg/ml BSA (Sigma). The medium was changed every day.

Western Blot Analysis. Cells were lysed in 63 mM Tris-HCl (pH 6.8), 10% glycerol, and 2% SDS. Bromphenol blue (0.05%) was added to samples of 12-μg protein each, and the samples were fractionated by 15% SDS-PAGE under nonreducing conditions. The proteins were subsequently transferred to a nitrocellulose membrane, which was incubated in a blocking solution (5% skimmed milk and 0.1% Tween 20 in Tris-buffered saline; *i.e.*, 50 mM Tris-buffered saline supplemented with 0.15 M NaCl) for 90 min at room temperature and subsequently washed in Tris-buffered saline. Thereafter, the membrane was exposed for 16 h at 4°C to a mouse antihuman VEGF antibody (1:500, R&D Systems, Minneapolis, MN), followed by a horseradish peroxidase-conjugated goat antimouse antibody (1:1000; DakoCytomation, Glostrup, Denmark). Bands were visualized using enhanced chemiluminescence (Amersham Biosciences, Uppsala, Sweden). Recombinant human VEGF165 and VEGF121 were used as controls (R&D Systems).

Immunofluorescence Detection of VEGF. Cell cultures were fixed in 4% formaldehyde in PBS for 20 min at 4°C and then processed for immunocytochemistry as described earlier (16). The cells were incubated with a monoclonal mouse antihuman VEGF antibody (dilution 1:100; R&D Systems), followed by a goat antimouse IgG Texas Red conjugate (1:200; Vector Laboratories, Burlingame, CA). Thereafter, the cells were rinsed in PBS and distilled water, and mounted in Vectashield medium (Vector Laboratories). The cultures were examined in a Nikon photomicroscope (Nikon Corporation,

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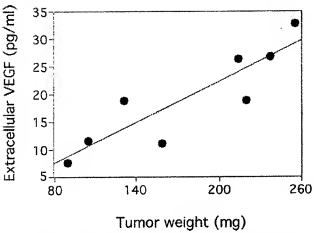

Fig. 1. Correlation between tumor weight and extracellular VEGF *in vivo* measured using microdialysis. Solid MCF-7 tumors in nude mice were subjected to microdialysis for collection of VEGF in the extracellular space. Tumor weight and extracellular VEGF correlated significantly ($r^2 = 0.788$; $P = 0.009$).

Tokyo, Japan) using green exciting light and a 590-nm barrier filter, and photographed with a digital camera. Controls incubated without anti-VEGF antibodies did not stain.

Animals and Ovariectomy of Mice. Female athymic mice (6–8-weeks-old) were purchased from M&B (Ry, Denmark). They were housed in a pathogen-free isolation facility with a 12-h-light/12-h-dark cycle, and fed with rodent chow and water *ad libitum*. The Linköping University animal ethics research board approved all of the animal work. Mice were anesthetized with i.p. injections of ketamine/xylazine and ovariectomized; 3-mm pellets containing 17β-estradiol, 0.18 mg/60-day release, or placebo pellets (Innovative Research of America, Sarasota, FL) were implanted s.c. in the back of the animal 7 days before tumor induction. The pellets provide continuous release of estradiol at serum concentrations of 150–250 pM (confirmed by serum analysis), which is in the range of physiologic levels seen in mice during the estrous cycle. One week after surgery, MCF-7 cells (5×10^6 cells in 200 μl PBS) were injected s.c. on the right hind flank. Tumor volume was determined by measuring length, width, and depth of the tumor every 5 days using a caliper. At a tumor size of ~300 mm^3, the mice were divided into two subgroups. One group continued with the estradiol treatment only, whereas tamoxifen (1 mg/every 2 days s.c.) was added to the estradiol treatment for 2 weeks in the other group. Plasma was collected in heparin by cardiac puncture.

Microdialysis Equipment and Experiment. Microdialysis has previously been used extensively for sampling of molecules from the extracellular space from various tissues *in vivo* (17). Tumor-bearing mice were anesthetized i.p. with ketamine/xylazine and were kept anesthetized by repeated s.c. injections. A heating pad maintained the body temperature. A small skin incision was made, and microdialysis probes (CMA/20, 0.5-mm diameter; PES membrane length = 10 mm, 100,000-molecular weight cutoff; CMA/Microdialysis, Stockholm, Sweden) were inserted into tumor tissue and fixed by sutures to the skin. The probes were connected to a CMA/102 microdialysis pump (CMA/Microdialysis) and perfused at 1 μl/min with saline containing 154 mM NaCl and 40 mg/ml dextran (Pharmalink, Stockholm, Sweden). After a 30-min equilibration period, the outgoing perfusate was collected on ice and stored at −70°C for subsequent analysis. We have validated recently the 100,000-molecular weight cutoff membrane for VEGF measurement in murine tumors (18) and also have shown that this technique is suitable for VEGF measurement in human breast tissue (19).

Immunohistochemistry of Tumor Sections. Formalin-fixed, paraffin-embedded tumors were cut in 3-μm sections, deparaffinized, and subjected to antihuman VEGF immunohistochemistry (monoclonal mouse antihuman VEGF; dilution 1:20; R&D Systems, with Envision detection; DakoCytomation) or anti-von Willebrand's factor (rabbit antihuman von Willebrand; dilution 1:1000; DakoCytomation). Sections were counterstained with Mayer's hematoxylin. Negative controls did not show staining. In a blinded manner, 10 high power fields (×200) were examined by section of three different tumors in each group. For VEGF scoring, the whole material was scanned to identify the range of intensity of the staining. Thereafter, the staining on the tumor sections was scored either as weakly or strongly positive. Vessel quantification of tumor sections was conducted as described previously using a Nikon microscope equipped with a digital camera (20). Percentage of area stained positively for von Willebrand's factor was assessed using Easy Image Measurement software (Bergstrom Instruments). Tumor sections were also subjected to H&E staining.

Quantification of VEGF Protein. Microdialysate, plasma samples, and cell culture medium were analyzed for VEGF using a commercial quantitative immunoassay kit for human VEGF (QuantGlo; human VEGF; R&D Systems) without preparation. Cell culture pellets were frozen at −70°C, thawed once, diluted in PBS, and sonicated for 10 s. Protein content was determined using the method described by Lowry *et al.* (21). According to the manufacturer, this kit measures the VEGF165 and VEGF121 isoforms. The sensitivity is <1.76 pg/ml, and intra-assay and interassay precision is 3–8%. The precision of the ELISA kit was confirmed during the experiments. All of the samples were assayed in duplicate.

Quantification of VEGF mRNA. VEGF mRNA was detected using human VEGF Quantikine Colorimetric mRNA Quantitation kit (R&D Systems). Cell lysate samples were made using the provided cell lysate diluent and frozen at −70°C. According to the manual, the whole cell lysate samples were hybridized with the VEGF probe and then transferred to a plate coated with streptavidin. The VEGF mRNA probe and calibrator in this kit cannot distinguish between the different isoforms of VEGF. VEGF mRNA was quantified and correlated to the total amount of protein in the samples.

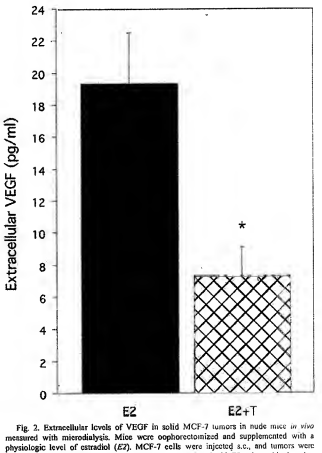

Fig. 2. Extracellular levels of VEGF in solid MCF-7 tumors in nude mice *in vivo* measured with microdialysis. Mice were oophorectomized and supplemented with a physiologic level of estradiol (*E2*). MCF-7 cells were injected s.c., and tumors were formed on the right hind flank. One group of mice continued with E2 only, and in the other group tamoxifen (*T*) treatment was added to the E2 treatment. Thereafter, microdialysis was performed on size-matched tumors as described in "Materials and Methods." The perfusate was analyzed using ELISA ($n = 8$ in the E2 group and $n = 5$ in the E2 + T group; *, $P < 0.05$).

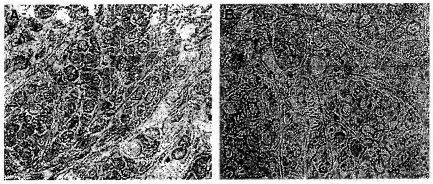

Fig. 3. Immunohistochemistry of VEGF in tumor sections. Mice were treated as described in Fig. 2. There were no differences in distribution or intensity of staining in tumors from the two different treatment groups. Tumor sections from three different tumors in each group and 10 randomly selected areas from each tumor were analyzed. Representative tumors are shown. *A*, tumor section from an estradiol-treated tumor. *B*, tumor section from an estradiol + tamoxifen-treated tumor.

Statistics. The values represent the mean ± SE. Statistical analyses were performed using Student's *t* test, ANOVA with Fisher's post hoc test, or Fisher's exact test where appropriate.

RESULTS

Tamoxifen Decreased Extracellular VEGF in Solid MCF-7 Tumors. MCF-7 tumors require estradiol for growth in nude mice. Therefore, there is no untreated control group or a tamoxifen-alone group in the *in vivo* experimental design. To explore the secretion of VEGF *in vivo*, we performed microdialysis to sample the extracellular fluid of the tumors. Because hypoxia is a potent regulator of VEGF expression, mainly through hypoxia-inducible factor-1, the first experiments were conducted on tumors of different sizes to explore whether tumor size influenced the released extracellular VEGF *in vivo* (22). As shown in Fig. 1, tumor size correlated significantly with extracellular VEGF ($r^2 = 0.788$; $P = 0.003$). Therefore, to avoid this confounding factor, all of the experiments were performed on size-matched tumors (in all of the treatment groups). Tumor weights were 176 ± 22 mg in the estradiol group and 171 ± 21 mg in the estradiol + tamoxifen group. Tumor sections did not reveal any necrotic areas on H&E staining. Microdialysis was performed on estradiol-treated tumors at days 35 and 50 after tumor cell injection, revealing no difference in the secreted VEGF over this timeframe in tumors of similar size. In tumors from mice treated with a combination of estradiol + tamoxifen for 2 weeks, the extracellular levels were 7.3 ± 1.7 pmol/ml; in tumors from mice treated with estradiol only, the levels were 19.4 ± 3.2 pmol/ml ($P < 0.05$; Fig. 2). The difference in the extracellular tumor levels of VEGF was not revealed in plasma of the mice. Plasma levels of VEGF in estradiol-treated animals were 10.5 ± 2 pM compared with 12 ± 0.4 pM in estradiol + tamoxifen-treated mice.

Immunohistochemistry of VEGF in Solid MCF-7 Tumors. Immunohistochemistry of intracellular cytoplasmic VEGF in tumor sections showed no detectable difference between the groups; in tumors from estradiol-treated animals, 22 of 30 sections were scored as strongly positive, whereas 19 of 30 were strongly positive in the estradiol + tamoxifen group ($P = 0.6$). Extracellular VEGF either bound to the cell surface or in the extracellular matrix was not possible to detect. This suggests that the intracellular content of VEGF was similar in the estradiol group and in the group treated with estradiol + tamoxifen. Representative tumor sections are shown in Fig. 3.

Tamoxifen Decreased Tumor Vasculature. To evaluate whether the decreased extracellular levels of VEGF had a biological relevance for tumor vasculature, we quantified vessel area stained with anti-von Willebrand's factor. We found that the vessel area was significantly lower on tumor sections from animals treated with a combination of estradiol + tamoxifen compared with estradiol treatment only (1.2 ± 0.2% of total area *versus* 5 ± 1.1%; $P < 0.05$; Fig. 4*C*). Representative tumor sections are shown in Fig. 4, *A* and *B*.

Tamoxifen Decreased VEGF Secretion into the Cell Culture Media but Increased Intracellular VEGF Protein. To confirm our *in vivo* results, we determined the amount of intracellular and secreted VEGF from MCF-7 cells in culture using a quantitative ELISA in a time course experiment. MCF-7 cells were exposed to the various treatments, and the media were changed every day. Cells and media were harvested on days 1, 3, and 7. Fig. 5*A* shows the secretion, which was increased by estradiol exposure on days 3 and 7, whereas estra-

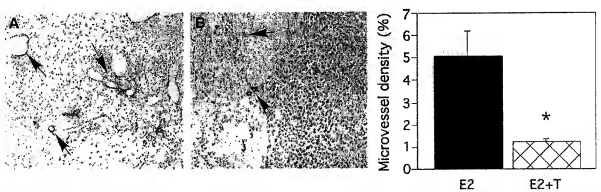

Fig. 4. Tamoxifen (*T*) decreased tumor vasculature. Mice were treated as described in Fig. 2. Tumor sections were stained with anti-von Willebrand's factor, and vessel area was counted on tumor sections. *A*, representative MCF-7 tumor exposed to estradiol (*E2*). *B*, representative MCF-7 tumor treated with a combination of E2 + T. *C*, tumor vessel density quantification was conducted in a blinded manner. Ten randomly selected areas of three different tumors in each group were counted (*, $P < 0.05$).

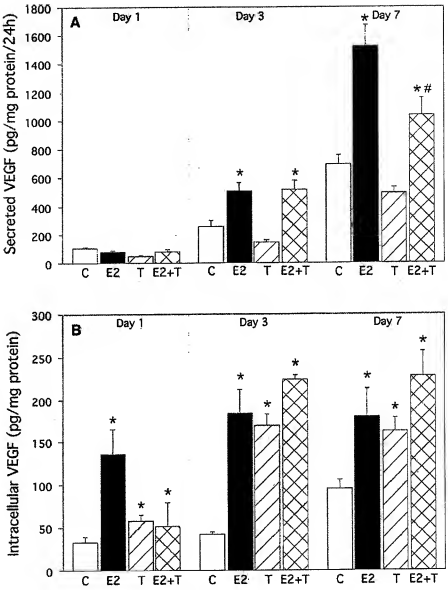

Fig. 5. Secreted and intracellular VEGF after hormone exposure of MCF-7 cells in culture. MCF-7 cells were cultured without hormones (C) or in the presence of estradiol (*E2*; 10^{-8} M), tamoxifen (*T*; 10^{-6} M), or a combination of E2 + *T*. The media were changed every day. VEGF was measured using ELISA after 1, 3, and 7 days in culture (n = 3–8 in each group; *, $P < 0.01$ compared with control cells; #, $P < 0.01$ compared with E2-treated cells).

diol + tamoxifen partially reversed the secretion at the same time points. However, the intracellular levels exhibited a similar increase in all of the hormone treatment groups compared with the control group (Fig. 5*B*).

Western Blot of VEGF. The ELISA kit we used in this study is not validated for measurement of cell culture lysates, and to confirm these results we also performed a Western blot analysis. The Western blot analysis confirmed our ELISA results and showed an increase of VEGF165 in all of the treatment groups compared with control cells (Fig. 6*A*). Three different Western blot analyses were scanned, and densitometry was performed ($P < 0.05$ between hormone-treated cells compared with control cells; Fig. 6*B*).

Estradiol and Tamoxifen Increased VEGF mRNA Levels. To confirm that there was a true increase in synthesis of VEGF and not an increased uptake, we performed mRNA quantification. All of the hormone treatments increased the VEGF mRNA levels ($P < 0.05$; Fig. 7).

Intracellular Localization of VEGF. To further investigate whether hormone treatment also affected the intracellular localization of VEGF, we performed immunofluorescence staining of the cells. As shown in Fig. 8, *B–D*, cells from all of the treatment groups exhibited similar intracellular cytoplasmic localization of VEGF and a more intense staining compared with control cells in Fig. 8*A*.

DISCUSSION

In this study we show for the first time that tamoxifen in combination with estradiol decreases extracellular VEGF, measured with microdialysis, in solid breast cancer tumors *in situ* in nude mice compared with estradiol treatment only. Our *in vivo* findings were verified *in vitro*, where extracellular levels of VEGF in cell culture media decreased significantly after exposure to a combination of estradiol + tamoxifen compared with estradiol exposure only. Immunohistochemistry of the same tumor sections did not reveal any

different staining between the groups. Similarly, *in vitro* tamoxifen increased intracellular VEGF protein and mRNA in a similar fashion as estradiol. It should be noted that the *in vivo* model does not contain a nonestradiol control group, but the estradiol and estradiol + tamoxifen groups exhibited similar results *in vitro* and *in vivo*.

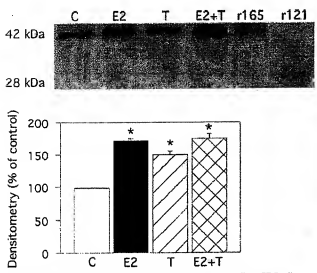

Fig. 6. Western blot analysis of intracellular VEGF in MCF-7 cells. MCF-7 cells were cultured as described in Fig. 5 and in "Materials and Methods." Recombinant VEGF165 and VEGF121 were used as controls. *A*, representative gel of three separate experiments. *B*, densitometry of three different gels (*, $P < 0.05$). An increase in VEGF was observed in all treatment groups in accordance with the VEGF levels quantified with the ELISA kit.

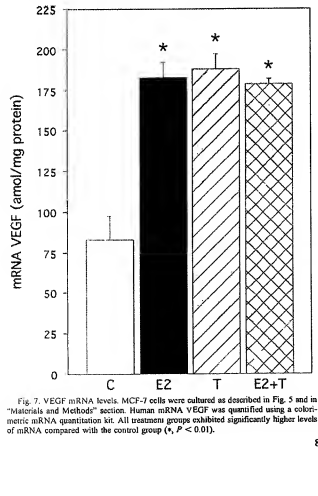

Fig. 7. VEGF mRNA levels. MCF-7 cells were cultured as described in Fig. 5 and in "Materials and Methods" section. Human mRNA VEGF was quantified using a colorimetric mRNA quantitation kit. All treatment groups exhibited significantly higher levels of mRNA compared with the control group (*, $P < 0.01$).

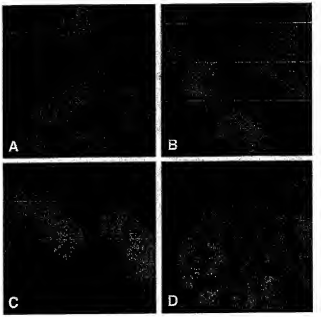

Fig. 8. Subcellular localization of VEGF in MCF-7 cells. MCF-7 cells were cultured as described in Fig. 5 and in "Materials and Methods." *A*, control cells without hormones. *B*, estradiol (*E2*) -exposed cells. *C*, tamoxifen (*T*) -exposed cells. *D*, E2 + T-exposed cells. Cells treated with hormones revealed a more intense staining compared with control cells, but no differences in subcellular localization of VEGF were observed.

Tamoxifen is the most widely used nonsteroidal antiestrogen for the treatment of hormonal-responsive breast cancer. It is well known that tamoxifen has estrogen antagonist/agonist modes of action, and several studies have shown that tamoxifen induces VEGF mRNA levels and intracellular protein (14, 15). This enhanced expression would seem to lead to an increased metastatic potential of cancer cells and is in conflict with clinical data, which show that tamoxifen prevents metastasis and increases overall survival of patients. However, there are, to our knowledge, no studies about effects of tamoxifen on extracellular levels of VEGF, the extracellular space being the biologically active site for VEGF.

The shorter isoforms of VEGF, 121 and 165, are soluble secreted proteins, although a portion of the 165 form remains bound to the cell surface, whereas the larger isoforms bind tightly to heparin and are sequestered in the extracellular matrix (23, 24). VEGFs are bioactive as freely diffusible proteins in the extracellular space, where they act on endothelial cells by stimulating cell proliferation, migration, and tubular organization and increase vascular permeability (24). Previous assessments of VEGF protein have been performed by immunohistochemistry or immunoassay of tissue extracts, and these measurements appear to correlate with microvessel density at least in invasive ductal carcinoma of the breast (25, 26). However, VEGF121, considered to be the most potent stimulator of angiogenesis *in vivo* and the predominate isoform in primary human breast cancer, diffuses freely into the extracellular space from the cells producing it and cannot be detected by immunostaining of tumor sections (7, 27). VEGF measured in blood has been considered as an alternative to these methods, but the interpretation of such studies has been complicated by the fact that most serum VEGF is derived from platelets, which are activated on coagulation (28). Unlike VEGF measured in serum, VEGF measured in plasma has been shown to be significantly higher in breast cancer patients compared with control patients, although the plasma levels do not appear to correlate with intratumoral VEGF assessed by immunohistochemistry (29). Moreover, in the same study tamoxifen treat-

ment was significantly associated with higher circulating and platelet-derived VEGF levels (29). We have shown recently that only ~45% of plasma levels of VEGF in tumor-bearing mice originate from the tumor and do not reflect extracellular VEGF secreted by the tumor (18). In line with these results, the plasma levels in the present study did not reveal the known differences in extracellular VEGF in the tumors detected using microdialysis.

Our results suggest that a regulation of extracellular VEGF may take place at a post-translational level. It is possible that tamoxifen reduces extracellular levels by blocking either the secretion or the release of stored pool of VEGF from the cell. In addition, it has been shown previously that the longer isoforms of VEGF may be converted into soluble, bioactive forms by proteolytic cleavage (24). This cleavage may be especially important in tumors in which the local microenvironment generally expresses a high proteolytic activity (30). It has been shown that increased activities of matrix metalloproteinases are associated with increased VEGF levels and increased tumor angiogenesis (31, 32). A direct measurement of VEGF locally in the tumor, such as that provided by microdialysis, might more accurately reflect the amount of extracellular, and, therefore, theoretically bioactive, protein released by the tumor. We have shown recently that microdialysis is a reliable technique for measurement of VEGF (18). Moreover, we have shown previously that microdialysis is applicable for human breast tissue in providing measurements of VEGF and other high molecular weight proteins and of low molecular weight compounds (19, 33–35).

In a recent study, it was suggested that tamoxifen treatment of nude mice with MCF-7 explants increased VEGF mRNA to levels above those observed with estradiol treatment. The authors interpret that the subsequently increased vascular permeability may lead to a decreased generation of functional microcapillaries (15). These results are in line with our intracellular results; however, the extracellular VEGF in that study was never analyzed, and the hormone treatments differed from ours. In that experiment, supraphysiologic levels of estradiol were used, and the tamoxifen-treated tumors contained large necrotic areas, suggesting that those tumors were highly hypoxic (15). Hypoxia is a strong inducer of tumor angiogenesis and VEGF (22, 36). Thus, hypoxia must be controlled to properly interpret the effects of various interventions, such as hormonal treatments, on VEGF. In our study, all of the tumors were size matched, and microscopic sections of the tumors did not reveal any necrotic areas. The mice in our study received supplements of 17β-estradiol at physiologic levels and were treated with tamoxifen at therapeutic levels because estrogen effects are related to the dose and type of hormone used. Estrogen often exhibits a bell-shaped dose-response curve; therefore, it is important to use physiologic levels of estrogen and naturally occurring 17β-estradiol in pathogenetic studies. Moreover, to mimic the clinical situation, at least for premenopausal women, we chose to give tamoxifen to the mice without discontinuing the supplemented estradiol.

In summary, we show, using microdialysis, that tamoxifen in combination with estradiol decreased extracellular levels of VEGF in solid MCF-7 tumors in nude mice *in vivo* compared with estradiol treatment only. These *in situ* results were verified *in vitro* in cell culture studies of extracellular VEGF release into the cell culture media. However, the intracellular levels of VEGF increased in a similar manner with treatments of either tamoxifen or estradiol. Estrogen and angiogenesis are important factors in breast cancer progression, and metastasis and antiestrogen treatments are cornerstones in breast cancer treatment. Therefore, additional investigations of hormonal regulation of angiogenesis and angiogenic factors in breast cancer are warranted. We believe that our results emphasize the need for studies on the regulation of proteins where they are biologically active—in the case of VEGF, in the extracellular space.

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